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REVIEW

DNA electrotransfer: its principles and an updated review of its therapeutic applications

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The use of electric pulses to transfect all types of cells is well known and regularly used in vitro for bacteria and eukaryotic cells transformation. Electric pulses can also be delivered in vivo either transcutaneously or with electrodes in direct contact with the tissues. After injection of naked DNA in a tissue, appropriate local electric pulses can result in a very high expression of the transferred genes. This manuscript describes the evolution in the concepts and the various optimization steps that have led to the use of combinations of pulses that fit with the known roles of the electric pulses in DNA electrotransfer, namely cell electropermeabilization and DNA electrophoresis. A summary of the main applications published until now is also reported, restricted to the in vivo preclinical trials using therapeutic genes.
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Introduction

Nonviral gene therapy using the combination of 'physical' approaches and naked DNA is rapidly developing for two main reasons: the use of naked DNA eliminates the limitations and the risks linked to the use of viruses (coding sequence length in the case of the adenovirus associated virus, insertional mutagenesis in the case of the retrovirus, immunological responses in the case of the adenoviruses, etc) and, in spite of extensive research, efficient and safe chemical vectors have not yet been developed for *in vivo* gene delivery.

There are several physical approaches for nonviral gene therapy. (i) The simplest, of course, is the injection of naked DNA, which in the skeletal or cardiac muscle leads to some expression of the injected genes;[1–3] however, this expression is very low and very variable from sample to sample. (ii) The hydrodynamic method consists in the very rapid injection through the mouse tail vein of a large volumes of DNA solution: it results in a very efficient transfection of liver cells, even though the procedure is somehow dangerous for the treated mice;[4–6] indeed, part of the mechanism is based on the transient heart failure resulting from the injection, which blocks the fluid distribution in the body and provokes a liquid overpressure in the liver.[4] (iii) For physical DNA transfer to superficial tissues like the skin or the leaves in the plant kingdom, the 'biolistic' approach ('jet injection' and 'gene gun') also leads to good transfection levels. The commonly termed 'gene gun' consists in a device propelling plasmid-coated gold microparticles.[7,8] For the 'jet injection' or 'needle-free' injection, the DNA is pushed at high pressure and high speed through a tiny orifice at the head of the injector, creating an ultrafine stream of high-pressure fluid that penetrates the skin.[9,10] (iv) The proof of the concept of sonoporation (use of focused ultrasounds to permeate the cells) has just been developed and still requires further elaboration.[11] (v) DNA electrotransfer has been used with success since 1998 and is becoming a real alternative to the viral methods for *in vivo* gene transfer.

The use of electric pulses is very popular for the transfection of bacterial and eukaryotic cells *in vitro*. The initial limitation of the so-called electroporation, a low cell survival, could be overcome by the use of appropriate electric pulses. The technique was then transferred *in vivo*, and termed DNA electrotransfer or electrogenetherapy. In this article, we present an historical survey of this approach, which will include the description of the bases of cell electropermeabilization and DNA electrotransfer, as well as several consecutive optimization efforts that have led to a very efficient and safe procedure. A summary of the main applications published until now is also reported, restricted to the *in vivo* preclinical trials using therapeutic genes.

The origin of DNA electrotransfer (the in vitro only period)

The first pioneering demonstration that DNA could be introduced into living cells by means of electric pulses was published by E Neumann in 1982.[12] He built a device with chambers specifically designed for the pulse delivery to the suspension of cells and DNA. More than 2 years were necessary before the publication of the second paper describing successful transfer of DNA to eukaryotic cells *in vitro* by H Potter in 1984.[13] Since this result

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was achieved using a classical (thus accessible) laboratory equipment, the ISCO 494 generator for proteins and DNA gel electrophoresis, many other groups could try this approach. The procedure consisted in creating a short circuit through the cell suspension, which caused the delivery of an exponentially decaying electric pulse to the cells. Since then, devices delivering exponentially decaying pulses have been developed by various companies.[14] However, already in 1985, J Teissié developed the first square wave pulse generator with outputs compatible with the needs for cell electropermeabilization *in vitro*.[15] In any case, since 1986, DNA electrotransfer is the most popular way to transfect bacterial cells and one of the good options for the *in vitro* transfection of eukaryotic cells as well.

Principles of the DNA electrotransfer

The exposure of living cells to short and intense electric pulses induces position-dependent changes in the transmembrane potential difference. These changes are well described by the equation of Schwann, which indicates that the value of the induced change is proportional to the cell radius and the scalar value of the external electric field (Figure 1). This change will superimpose to the resting transmembrane potential. When the transmembrane potential difference net value (the sum of the vectorial values of the induced and resting potential differences) is greater than 0.2–0.4 V, transient permeation structures are generated at the cell membrane level, because the membrane structure cannot resist the electrocompressive forces due to this potential difference. Electropermeabilization is thus a threshold phenomenon, imposed by the need to overpass a threshold value of the transmembrane potential difference.

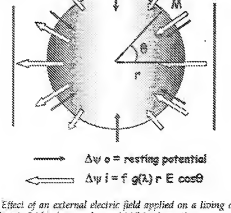

Figure 1 Effect of an external electric field applied on a living cell. The external electric field induces a change (Δψi) in the resting transmembrane potential (Δψo) of the cell. The value of the induced change depends on the shape of the cell (f) and the conductivity of the media g(λ). At point M on the cell surface, it is also proportional to the cell radius (r), the scalar value of the external electric field (E) and the cosinus of the angle θ (polar coordinate of point M).

The structure of these transient permeation structures is not yet elucidated. Some models proposed the generation of 'electropores' (described as 'holes' in the membranes), but cell electropermeabilization can be totally reversible and the theory hardly explains the resealing of these 'electropores'. Recent modelization by molecular dynamics[16] has suggested that under transmembrane potential differences, much larger than those necessary to obtain the 'physiological' reversible cell electropermeabilization, pores could indeed be generated. Still, the reversible structure remains undefined.

Properties of cell electropermeabilization: cell membrane crossing

Cell electropermeabilization is a general phenomenon that can be obtained in the cells of archaebacteria, eubacteria and eucaryota phyla. Indeed, all living cells are limited by a nonconductive membrane that isolates the internal conductive medium from the external medium. Thus, all cells will react to external electric fields by the induction of a transmembrane potential difference that, above a threshold value, will provoke the membrane destabilization. Under appropriate electrical parameters, this destabilization will be totally reversible, ensuring the survival of the transiently permeabilized cell. Owing to the use of very short pulses, cell electropermeabilization is a nonthermal phenomenon: this characteristic contributes to its reversibility (no denaturation of the membrane proteins, even though one of the cell electropermeabilization early models, which was not validated, considered protein denaturation as the *primum movens* of the membrane properties changes).[17] Finally, the most interesting property of the cell electropermeabilization, which supports several biomedical and biotechnological applications, is the fact that cell electropermeabilization allows the direct delivery of nonpermeant molecules inside the cell cytoplasm, bypassing the normal internalization route for these molecules (usually the endocytosis pathway). Small nonpermeant molecules can enter the electropermeabilized cells by diffusion through the transiently permeabilized cell membrane, while large nonpermeant molecules like DNA enter by other mechanisms as discussed below.

In vivo delivery of electric pulses

Before the *in vivo* delivery of electric pulses in the frame of the electrogenetherapy, other applications of the *in vivo* cell electropermeabilization were developed. In 1989 and 1990, Grasso *et al*[18] Grasso and Heller[19] applied electric pulses *in vivo* to rabbit cornea, in order to fuse human Hela cells to the cells of the cornea. Cell electrofusion, *in vitro*, is the consequence of the electropermeabilization of two adjacent cells or of the fact that two previously electropermeabilized cells are brought in close contact. In 1988, it was found that bleomycin toxicity is several hundreds of thousands times higher on electropermeabilized cells than on cells unexposed to the electric pulses.[20] This increase in toxicity was also found in preclinical experiments in which permeabilizing electric pulses were delivered transcutaneously to

transplanted[21] or spontaneous tumours in mice.[22] Moreover, because of the antitumour efficacy of this approach, clinical trials were rapidly performed.[23,24] This approach was termed electrochemotherapy, and the pulse conditions, used in almost all the published clinical trials,[25–27] were eight identical square wave pulses of 100 µs and 1300 V/cm at a repetition frequency of 1 Hz using external electrodes (transcutaneous pulses). These trials demonstrated that it is possible to deliver *in vivo* electric pulses to animals and patients and they greatly facilitated the development of the *in vivo* DNA electrotransfer.

Indeed, in parallel, Jon Wolff showed in 1990[1] that direct injection of naked DNA in skeletal muscle *in vivo* results in gene expression at low and variable levels. Thus, it seemed tempting to combine the injection of DNA and the electric pulses delivery.

In 1991, Titomirov *et al*[28] delivered exponentially decaying short pulses to skin after myc and ras genes injection and they were able to recover a few growing cells expressing myc and ras proteins that could, eventually, reflect the *in vivo* electrotransfer of these oncogenes. In 1996, Heller *et al*[29] delivered electrochemotherapy-type trains of short pulses (100 µs) to the liver after the injection of reporter genes DNA, with good levels of transfection that nowadays could also be partly explained by the injection itself, taking into account the results of the hydrodynamics method. In 1998, four groups, in three different tissues, consistently demonstrated good transfection levels using long pulses (5–50 ms): MP Rols and J Teissié in tumours,[30] Suzuki *et al* in the liver,[31] and Aihara and Miyazaki,[32] and Mir *et al*[33] in the skeletal muscle. The use of trains of several identical pulses in the milliseconds to tens of milliseconds duration range actually results in a highly significant increase in the level of expression of the reporter genes coded by the naked DNA injected in the target tissues. Later on, Lucas and Heller[34] compared short and long pulses, demonstrating that the level of expression was higher, and expression duration longer, when long pulses were delivered into the tissues.

Use of trains of identical electric pulses for efficient DNA electrotransfer in skeletal muscle

The earliest and most exhaustive series of experiments allowing to understand the mechanisms of DNA electrotransfer as well as to optimize such trains of identical electric pulses were reported in 1999.[35] Experiments analysed the respective influence of the pulse duration, voltage applied (or more precisely of the applied voltage to electrodes distance ratio), number of pulses and repetition frequency. Using the gene coding for the firefly luciferase, an increase of 200 times of the expression with respect to the naked DNA injection alone was shown , a large decrease in the variability of this expression, as well as a long-term expression since the high level of expression remained stable for at least 9 months. In the mouse skeletal muscle, using external electrodes (transcutaneous electric pulses) and trains of identical electric pulses, optimal conditions are eight pulses of 20 ms and 200 V/cm at a repetition frequency of 1 or 2 Hz, delivered after the intramuscular injection of the DNA).[36,37] These conditions have been adapted to other tissues: in tumours, transfection levels that depended on the tumour type were found maximal using eight identical pulses of 20 ms and 500 or 600 V/cm at a repetition frequency of 1 or 2 Hz,[38] 250 V/cm in the liver,[39] and 500 or 750 V/cm for the skeletal muscle in neonate mice (7–10 days old mice).[40]

Roles of the electric pulses in DNA electrotransfer

Experiments showed that, after the electric pulses delivery, tissues remain permeabilized for several minutes.[35,41,42] Moreover, for an efficient transfer, DNA must be injected before the electric pulses delivery. Thus, permeabilization of the cells is not sufficient even though it is necessary since efficient electrotransfer requires sufficiently intense electric fields (above cell permeabilization threshold).[36,37] Moreover, efficient electrotransfer requires sufficiently long pulses. The mechanism of DNA electrotransfer could not be just cell electropermeabilization and DNA diffusion through the permeabilized plasma membrane.

The role of the electric pulses in DNA electrotransfer has then been studied using combinations of pulses. Instead of delivering trains of eight identical pulses (of 20 ms and 200 V/cm, at a repetition frequency of 1 Hz), cells were exposed to:

- 1 HV (high voltage, short pulse) of 100 µs at 800 V/cm (an electrochemotherapy-like pulse, with a field strength adapted to the skeletal muscle, the muscle fibres having a diameter larger than the average diameter of the tumour cells; using eight of such pulses at 1 Hz repetition frequency, Gehl *et al*[37] showed that this field strength was the highest that one could deliver to the muscle fibres without provoking their irreversible electropermeabilization).

followed by

- 1 or several LV (low voltage, long pulse) of 100 ms at 80 V/cm (nonpermeabilizing pulses, of a field intensity below the threshold for reversible permeabilization in the mice skeletal muscle).[43]

The first experiments were performed using two classical square wave generators, with LV of 83 ms, a limitation imposed by the devices used, and a manual switch to deliver the LV(s) after the HV.[43] Then a device for the controlled generation of such combinations of HV and LV pulses, with, moreover, a controlled gap between the HV and LV pulses, was prepared by the Faculty of Electrical Engineering of the University of Ljubljana, based on a previously developed electroporator.[44] With such an equipment, the roles of the electric pulses in DNA electrotransfer could actually be analysed.

The efficacy of several combinations of pulses (1 HV alone, or 1 HV followed by 1 LV, or 1 HV followed by 4 LV) was compared.[42] It was shown that the duration of the high permeabilized state of the muscle fibres was the same for the three combinations tested, all of them including the same HV pulse. On the contrary, the authors found that efficacy was only achieved if at least

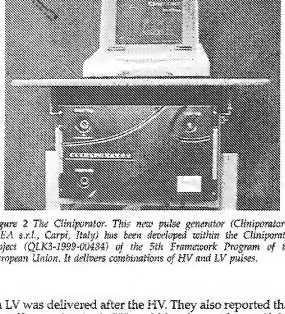

Figure 2 The Cliniporator. This new pulse generator (Cliniporator™, IGEA s.r.l., Carpi, Italy) has been developed within the Cliniporator project (QLK3-1999-00484) of the 5th Framework Program of the European Union. It delivers combinations of HV and LV pulses.

an LV was delivered after the HV. They also reported that the efficacy of a single LV could be observed even if this LV was delivered up to 100 s after the HV, while in the case of the delivery of 4 LV, efficacy could be achieved even if the 4 LV were delivered up to 3000 s after the HV. Since almost no efficacy was found with the LV alone, the conclusions of the authors were that the HV pulses delivery (permeabilizing pulses) were mandatory, but that the efficacy of the procedure was brought by the LV pulses. Other arguments have contributed to point out that the long LV pulses act on DNA, provoking its electrophoretic displacement.[45]

The electric pulses thus have two roles, the 'electroporation' of the target cells and the electrophoretic transport of the DNA 'towards or across' the cell membrane. Target cell electropermeabilization is mandatory, but the electrophoretic component of the electric pulses is actually instrumental in DNA electrotransfer efficacy.

These combinations have been studied within the Cliniporator project (QLK3-1999-00484) of the 5th Framework Program of the European Union. Moreover, a new pulse generator (Cliniporator™, IGEA s.r.l., Carpi, Italy), able to deliver these combinations of pulses, has been developed within this project (Figure 2). In the skeletal muscle and the skin, appropriate pulses parameters have led to a further increase of the expression of the luciferase coding plasmid with almost no histological modification.

Interests of DNA electrotransfer

In vitro, DNA electrotransfer is interesting because it is based on cell electropermeabilization, which is the perturbation of cell membrane impermeability by physical means, with neither addition nor withdrawal of membrane components as it happens when chemical permeabilization means are used. Thus, full recovery is facilitated. Moreover, if actually controlled, cell electropermeabilization is a nonthermal effect, without protein denaturation (which also facilitates cell recovery). Moreover, the method is simple, since it only requires to mix the cells and the DNA and to pulse the mixture.

In vivo, DNA electrotransfer is also interesting because it allows the transfer of genes into tissues without using virus. Moreover, no chemical method works *in vivo* better than the direct electrotransfer of the naked DNA. The method is also very rapid: the new constructs made by usual molecular biology approaches can be amplified by rapid 'minipreparations' of DNA and quantified by optical density determination; then it is sufficient to adjust plasmid concentration, to inject, and to 'pulse' (with viral methods, constructs must be inserted in a viral background, transfected in producing cells, and then virions must be produced, collected, isolated, concentrated and titrated before they could be injected).

Therapeutical applications already developed in preclinical trials

Tables 1 and 2 summarize the result of an extensive search for publications reporting the *in vivo* delivery of genes of therapeutic interest by means of DNA electrotransfer. Publications using only reporter genes have not been included in the tables. Experiments have been classified according to the main applications foreseen by their authors.

Most of the experiments deal with gene transfer to the skeletal muscle in mice. However, gene transfer to tumours, brain, lumbar intrathecal space, skin, liver, cornea, brain, penile corpora cavernosa and seminiferous tubes have also been reported. Experiments, mainly in mice (about 80% of the publications) have also been performed in rats, pigs, rabbits, guinea pigs, sheep, goats, dogs and cattle. These experiments also demonstrate the safety of the procedure, the possibility to repeat the treatment (shown already in 1999 by Rizzutto *et al*[120]), as well as the possibility to coelectrotransfer up to three plasmids into the same skeletal muscle fibres.[88] The main general application is immunotherapy (48%; 54/113). Cancer treatment (38%; 43/113), metabolic disorders or metabolism modification (17%; 19/113) and correction of organ or site-specific diseases (14%; 16/113) are the three other frequent applications. Monogenetic diseases (9%; 10/113), cardiovascular diseases (9%; 10/113) and analgesia (2%; 2/113) are other applications also found in the literature.

It must be noted that each of these applications includes the use of a large variety of genes. In this respect, it is necessary to point out that the genes of proteins involved in the immune system responses have been the most usually transferred genes for vaccination, cancer, treatment of arthritis, immunological protocols, etc. These genes include those coding for the IL-2, IL-4, IL-10, IL-12, IL-18, IL-18 binding protein, soluble TNF receptor, GM-CSF, tumour epitopes, the HIV gag gene, recombinant monoclonal antibodies, mycobacterial antigens, etc. The details are listed in the Table 2. In fact, this observation must be related to the fact that in many cases the transfected tissue is the skeletal muscle, used as a cell factory for the production of factors that will act systemically on distant targets.

Therapeutic levels have been achieved. For example, in the case of the gene coding for the erythropoietin

Table 1 Applications of the *in vivo* delivery of genes of therapeutic interest by means of DNA electrotransfer

Applications[a]	*Diseases*[a]	*Genes*	*Tissues*	*Animals*	*Ref.*
Analgesia 2	Analgesia 2	POMC (proopiomelanocortin)	Intrathecal space	Rat	46, 47
Cancer 43	Cancer 43	IL-2; IL-12; IL-18; INF α; GM-CSF; CpG containing DNA; full TRP-2 or epitopes; diphtheria toxin; HSV TK; TIMP; p53; bcl-xs; MBD-2; antisens VEGF; Flk-1 VEGF receptor; metargidine (MDC-15); Stat3 variant; K1-5; K1-3-HAS; endostatin	Muscle, tumour, skin, liver	Mouse, rat	34, 48–87, 101, 102
Cardiovascular diseases 10	Atherosclerosis 2	IL-12; Human plasma platelet-activating factor acetylhydrolase (PAF-AH)	Muscle	Mouse	88, 89
	Ischaemia 4	IL-10; IL-18; NF-6B binding sites containing DNA; hVEGF-A and hVEGF-B, protein-disulfide isomerase	Muscle, right hippocampus	Mouse, rat	90–93
	Myocarditis 4	IL-1-ra ; IL-10	Muscle	Mouse, rat	94–97
Immuno-therapy 54	See Table 2	See Table 2	See Table 2	See Table 2	
Metabolic disorders 19	Anaemia 10	Epo; dimeric erythropoietin fusion protein	Muscle, skin	Mouse, rat	113–122
	Diabetes 6	IL-4; insulin precursors; codelivery of B7-1 and PPins or CEA; IGF-I	Muscle	Mouse	123–128
	Neuropathy 1	NT3 neurothrophin3	Muscle	Mouse	129
	Thrombocytopenia 2	Recombinant human thrombopoietin (rhTpo)	Muscle	Mouse	130, 131
Monogenetic diseases 10	β-Thalassemia 2	Epo	Muscle	Mouse	132, 133
	Haemophilia B 1	Factor IX	Muscle	Mouse, dog	134
	Mucopolysaccharidosis 1	IDS (iduronate-2-sulphatase)	Muscle	Mouse	135
	Myodystrophy 5	Dystrophin or minidystrophin; laminin α2; GA-binding protein	Muscle	Mouse	136–140
	Neuron degeneration 1	Cardiothrophin	Muscle	Mouse	40
Organ- or tissue-specific diseases 16	Arthritis 6	IL-10; POMC (proopiomelanocortin); soluble TNF Receptor	Muscle	Mouse, rat	95, 141–145
	Bone formation 1	BMP-4	Muscle	Mouse	146
	Bronchopulmonary hyperreactivity 1	IL-10	Muscle	Mouse	112
	Erectile dysfunction 1	Neuronal NOS (nitric oxide synthase), penile NOS	Penile corpora cavernosa	Rat	147
	Gastric disorders 1	Gastrin	Muscle	Mouse, rat	148
	Kidney regeneration 1	HGF	Muscle	Rat	149
	Liver regeneration 2	HGF	Muscle	Mouse, rat	150, 151
	Muscle regeneration 2	IGF-1	Muscle	Mouse	126, 152
	Ocular diseases 1	Human tPA (tissue plasminogen activator)	Corneal endothelium	Rat	153
	Spermatogenesis rescue 1	Stem cell factor (SCF) cytoplasmic domain	Seminiferous tubules	Mouse	154

[a]Number in bold letters corresponds to the number of publications concerning the application or disease.

Table 2 Details of the applications involving immune system-related genes (cytokines, antigens, etc)

Applications[a]	*Disease*[a]	*Genes*	*Tissues*	*Animals*	*Ref.*
Cancer 27	Cancer 27	IL-2; IL-12; IL-18; INF α; CpG containing DNA; GM-CSF; FLT3-L; Rlak; TRP-2 or epitopes	Muscle, tumour, skin	Mouse	34, 48–70, 76, 101, 102
Cardiovascular diseases 7	Atherosclerosis 1 Ischaemia 2 Myocardiopathy 4	IL-12 IL-10; IL-10	Muscle Muscle	Mouse Mouse	88 88, 90
Metabolic disorders 2. Organ- or tissue-specific diseases 6	Diabetes 2. Bronchopulmonary hyperreactivity 1 Arthritis 5	IL-4; Codelivery of B7-1 or B7-1vva IL-10	Muscle Muscle	Mouse; rat Mouse	123, 125 112
Vaccination 12		IL-10; soluble p75 TNF receptor linked to the Fc portion of human IgG (sTNFR-Fc) INF α; IFNbeta; HA, influenza virus; HIV gag; Japanese encephalitis virus; mycobacterial antigens; recombinant mAb; chains of the 7g10 mouse mAb; chimeric hepatitis C virus envE2 glycoprotein; Bio 43	Muscle Muscle, skin	Mouse, rat Mouse, pig, sheep, goats, cattle, rabbit, guinea pig	95, 142–143 98–100, 103–111

[a]Numbers in bold letters correspond to the number of publications concerning the application or disease.

(epo), hematocrit increase has been achieved in many cases (Table 1). Expression of electrotransferred mini-dystrophin gene in the altered myodystrophic muscles of the mdx mice has been demonstrated (Table 1). The electrotransfer of the genes coding for antioangiogenic factors has demonstrated distant antitumour effects as well as antimetastatic effects in the murine model consisting in the intravenous injection of B16F10 cells.[83–85] Noticeable concentrations of the cytokines Interleukin-2 and GM-CSF in tumours transfected with the corresponding genes have been measured.[59] The biological effects observed in the publications listed in Table 1 demonstrate the efficacy of the electrogenetherapy for the treatment of various diseases.

Finally, DNA electrotransfer has also been used for biotechnological purposes. For example, the electrotransfer of the human erythropoietin gene in the oviduct of laying hens has also been carried out for the production of the human erythropoietin,[185] and the transfer of the growth hormone-releasing hormone has also been successfully performed in pigs, not for the treatment of a pig disease but for the achievement of an enhanced weight gain and improved body composition.[186–188]

Conclusion

In conclusion, DNA electrotransfer or electrogenetherapy constitutes a real alternative to viral approaches for gene transfer *in vivo*. Its efficacy is proven and there is no doubt on its biological safety. Moreover, DNA preparation is easy and secure, the roles of the electric pulses are described, the control of transfer conditions is achievable and appropriate equipment is available.

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